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RAPID IDENTIFICATION OF DENGUE VIRUS SEROTYPES USING MONOCLONAL ANTIBODIES IN AN INDIRECT IMMUNOFLUORESCENCE TEST (u)

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Dengue and dengue hemorrhagic fever occur in epidemic and endemic form throughout tropical areas of the world. Dengue virus types 1, 2, and 3 have been associated with epidemics of major impact in the Western Hemisphere since the 1960's (1-3) and in 1981 dengue type 4 was identified in the Carribean for the first time (4). Extensive serological crossreactions occurring among dengue virus serotypes as well as with other flaviviruses in commonly employed serological tests (i.e., hemagglutination-inhibition, immunofluorescence, and complement fixation) frequently interfere with identification of the antigenic type of dengue virus present in epidemic or endemic areas (5-8). Presently, the only certain method of identification is through the use of rigidly standardized reference antiserum in a virus plaque-reduction neutralization assay (3,9,10). Few laboratories possess sufficient resources to perform this test with the slowly replicating dengue viruses.

Monoclonal antibodies that were produced using the recently established hybridoma technology (11,12) have been used successfully to characterize viral antigens (13,14,15). The present study had as its objective the development of highly specific monoclonal antibodies suitable for rapid serotype identification of low passaged or unpassaged dengue virus from humans or insects using an indirect immunofluorescence test.

MATERIALS AND METHODS

Cell cultures and media.

LLC-MK2 (monkey kidney) cells were grown in medium 199 supplemented with 20% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 ug/ml). The C6/36 clone of Aedes

albopictus cells was grown in Earles minimum essential medium containing 10% FBS, penicillin, and streptomycin. Maintanance medium for infected cell cultures consisted of the appropriate growth medium containing 0.4% bovine plasma albumin instead of FBS.

Virus

Prototype dengue strains used in this study included DEN-1(Hawaii), DEN-2(New Guinea C), DEN-3(Philippines H87), and DEN-4(Philippines H241). Other members of the flavivirus group included Banzi(SAH336), Ilheus, Japanese encephalitis(M1/311), Kunjin(MRM-16), Langat(TP-21), Ntaya, St. Louis encephalitis(TBH-28), West Nile(E-101), Yellow fever(French neurotropic and 17D strains), and Zika. Two Sandfly Fever viruses (213452 and Candiru) were included as non-flavivirus controls. Virus inocula for cell culture studies were derived from routinely passaged virus-infected mouse brain stocks passaged 1 to 4 times through C6/36 cells.

Virus isolates were generously provided by Dr. Esmie Rose and Dr. P. Prabhaker (University of the West Indies, Kingston, Jamaica), Dr. A. Dyer (Ministry of Health, Jamaica) and Dr. J.P. Digoutte (Institute Pasteur, Dakar, Senegal). Virus isolates from the Carribean were provided as first passage isolates (Aedes pseudoscutellaris cells, AP-61) or human serum from recent dengue virus patients. African isolates were provided as acetone-fixed AP-61 cells on 12-well fluorescent antibody slides prepared at the Institute Pasteur, Dakar, and then tested by one of us (EAH) as described below during a visit there.

Virus isolates were processed by inoculating two LLC-MK2 cell cultutres and one C6/36 cell culture with 0.2 ml of each specimen. One LLC-MK2 cell culture was reserved for direct plaquing using an agar overlay; the other flasks were observed daily for cytopathic effects (CPE). After 7 days the flask reserved for direct plaquing was stained using a neutral red agar overlay and aliquots were removed from the other flasks for plaque assay of virus yields. When fifty percent of the cells showed CPE (7 to 14 days), cell culture supernatants were assayed for virus yield and then the infected cells were processed for immunofluorescence.

Hyperimmune mouse ascitic fluids (HMAF)

The preparation of HMAF containing reference antibodies directed against each of the prototype dengue viruses as well as selected arboviruses used in this study was described previously (16).

Monoclonal antibodies

Monoclonal antibodies directed against dengue virus antigenic

determinants were prepared by fusing P3x63Ag8 mouse myeloma cells with spleen cells from mice immunized with dengue virus antigens as described previously (17). Antibodies secreted by the hybridomas were detected by solid phase radioimmunoassay (RIA) (18). RIA-positive hybridomas which were reactive by the immunofluorescence assay (IFA) and sometimes by the hemagglutination-inhibition test (HAI) (5) or the plaque-reduction neutralization test (PRNT) (9,10) were selected for cloning, ascites production, and further study. Ascitic fluids were generally used in these studies because they contained on the average 100-to-1000 times more anti-dengue antibody than hybridoma tissue culture supernatants as detected by solid phase radioimmunoassay.

Indirect immunofluorescence assay (IFA)

Uninfected and infected cells displaying CPE were harvested by scraping each monolayer into 5 ml of Hank's balanced salt solution (HBSS) and the cells were collected by centrifugation at 1500 rpm for 10 minutes at 5°C. The cells were washed again with HBSS and phosphate-buffered saline, pH 7.4 (PBS), resuspended in PBS to 100,000-500,000 cells per ml, and spotted at 80 to 100 cells per 250x microscopic field on 15-well or 12-well fluorescent antibody slides. The slides were allowed to air dry, and the cells were fixed with ice cold acetone for 5 minutes. These slide preparations were frozen at -70°C for up to 6 months before use. Before staining, each slide was allowed to thaw at room temperature for 5 minutes, washed with PBS and drained. The cells were first incubated at 35°C with an appropriate dilution of hybridoma-derived ascitic fluid, DEN-2 HMAF which reacts with all four dengue serotypes, flavivirus HMAF (equal portions of DEN-2, Yellow fever, St. Louis encephalitis, Japanese encephalitis, and Banzi virus HMAF) or normal ascitic fluid. All monoclonal antibody preparations were screened first at a dilution of 1:10 with arbovirus-infected LLC-MK2 cells. Monoclonal antibodies used for serotyping were used at a dilution equal to 4 times the last dilution giving maximum fluorescence (4 fluorescence units; generally 1:20 to 1:80). Hyperimmune fluids were used routinely at a 1:40 dilution. After 30 minutes of incubation at 35°C, the slides were washed three times with PBS for 10 minutes each. The cells were then treated with fluorescein-conjugated goat anti-mouse serum for 30 minutes at 35°C, washed as above, mounted and viewed with a 25x oil immersion objective using a fluorescent microscope with epi-illumination.

Detection of DEN-2 virus replication in cell culture

LLC-MK2 or C6/36 cells grown to 90% confluency in 25 cm^2 flasks were infected with DEN-2(New Guinea C) at 1 plaque forming unit per cell. Flasks were observed daily for CPE and harvested at 0, 12, 24, 36, 48, 96, 120, and 144 hours postinfection (p.i.). Tissue culture supernatants

were titrated for virus yields and hemagglutination while the infected cells were processed for immunofluorescence using monoclonal antibodies from hybridoma 3H5 (type-specific) and polyclonal DEN-2 HMAF (flavivirus group reactive).

RESULTS

Characterization of monoclonal antibodies

In order to determine the specificity of ascitic fluids produced using selected hybridomas, each antibody preparation was tested against LLC-MK2 cells infected with each of the four dengue virus serotypes as well as with representative arboviruses. Representative results are presented in Table 1. Four patterns of specificity were discerned: flavivirus group reactive, dengue complex specific, dengue subcomplex specific, and dengue serotype specific. Flavivirus group reactive-antibodies reacted with cells infected with flaviviruses while dengue complex-specific antibodies stained cell infected only with dengue viruses. Dengue subcomplex-specific antibodies reacted only with cells infected with DEN-1 or DEN-3 while type-specific antibodies reacted only with the homologous dengue virus serotype against which they were prepared. These patterns of specificity are detailed below.

The majority of the antibody preparations produced were flavivirus group reactive. These include 1B8, 1B10, 1C10, 4G2, 2C4, 1B6, 4B10, and 1D7 which generally reacted with all of the flaviviruses tested and did not recognize the sandfly viruses of the Phlebovirus genus (Table 1).

Several monoclonal antibody preparations reacted solely with dengue virus serotypes. Two preparations (13E7 and 2F3) recognized only DEN-1 and DEN-3 virus infected cells (Table 1). One hybridoma was derived using DEN-1 antigens (13E7) while the other (2F3) was derived using DEN-3 antigens. Two other preparations (9D12 and 2H3) recognized all the members of the dengue complex. Hybridoma 9D12 was derived from DEN-1 immunized mice and 2H2 was derived from DEN-4 immunized mice (Table 1).

Nine hybridomas produced antibodies which were type specific by immunofluorescence for the virus serotype against which they were prepared. These include 15F3 and 5C11 (DEN-1); 3H5, 3H1, 2H3, 1C7, and 1C12 (DEN-2); 5D4 (DEN-3) and 1H10 (DEN-4). One of these preparations (3H5) was also type specific by PRNT, and five preparations were type specific by hemagglutination-inhibition: 3H1, 2H3, 1C7, 1C12 (DEN-2) and 1H10 (DEN-4) (16, data not shown).

Cytological distribution

The fluorescence pattern observed with monoclonal antibodies in infected LLC-MK2 cells was predominantly of the perinuclear type and was

Table 1.
Specificity of Monoclonal Antibody
Prepared Against Dengue Virus Antigens

Immunogen Serotype	Anti-Dengue Hybridoma			
	Type Specific	Dengue Subcomplex Specific[1]	Dengue Complex Specific[2]	Flavivirus Group Reactive
Den-1	15F3	13E7	9D12	
		5C11		
Den-2	3H5			1B8
		3H1		1B10
		2H3		1C10
		1C7		4G2
		1C12		2C4
Den-3	5D4	2F3		1B6
Den-4	1H10		2H2	4B10
				1D7

[1] DEN-1/DEN-3 cross
[2] Reacts with all four serotypes

either solid or coarsely reticular similar to that described by Cardiff et al using polyclonal antibodies (19). Infected cells which reacted with type-specific antibody generally displayed perinuclear fluorescence with fine granules which permeated portions of the cytoplasm. The fluorescence pattern observed with crossreactive antibody was perinuclear but usually involved more of the cytoplasm and was less granular in appearance. The intracellular fluorescence pattern observed with C6/36 cells was difficult to discern because of the the small size of the cells.

Temporal appearance of dengue antigens as detected by monoclonal antibodies

In order to correlate fluorescence reactions with biological events during virus replication, DEN-2 infected LLC-MK2 and C6/36 cells were harvested as described and reacted with type-specific monoclonal antibodies (3H5) and flavivirus group reactive antibodies (DEN-2 HMAF) in an indirect immunofluorescence test. These results are compared in Table 2 with the appearance of CPE, infectious virus, and viral hemagglutinin (HA). The appearance of CPE was detected in both LLC-MK2 and C6/36 cells by 36 hours p.i. with increasing degeneration of the cell monolayers throughout the course of the virus growth period. In LLC-MK2 cells the CPE consisted primarily of increasing amounts of rounded, pinocytotic, floating cells. Similar CPE as well as increasing amounts of polykarocytosis which reached a maximum (complete monolayer fusion) by 72 hours p.i. was seen in C6/36 cells. Infected, fused C6/36 cell monolayers gradually degenerated until few intact cells remained after 96 hours. Virus yields in both cell lines rapidly increased after 12 hours postinfection with maximum titers occurring 48 to 96 hours postinfection in LLC-MK2 and C6/36 cells, respectively. Virus yields in C6/36 cells averaged 3.3 $\log_{10}$ greater than LLC-MK2 cells throughout the multiple cycle replication curve. Increases in HA titers generally paralleled increases in virus titer; however, HA titers in LLC-MK2 cells decreased after 96 hours, while HA titers in C6/36 cells continued to rise. The detection of viral HA preceded the appearance of CPE by 24 hours in C6/36 cells.

Fluorescence patterns obtained using type-specific and flavivirus group reactive polyclonal antibodies were similar to those described previously. In LLC-MK2 cells the detection of viral antigens by immunofluorescence generally preceded the appearance of viral HA by 12 to 24 hours (Table 2). However, in C6/36 cells the detection of immunofluorescence was nearly simultaneous with the appearance of viral HA. In both cell lines polyclonal DEN-2 HMAF detected dengue antigens by 12 hours p.i. and with generally brighter fluorescence than that obtained using monoclonal antibodies.

TABLE 2

Temporal Appearance of Dengue-2 Antigens as Detected by Monoclonal Antibodies.

Cell Line	Hours p.i	CPE[1]	Virus Yield pfu/ml[2]	Hemagglutination Titer[3]	Immunofluorescence Reactions[4] 3H5	DEN-2 HMAF
LLC-MK$_2$	1	0	2.1×10^2	0	-	-
	12	0	0	0	+/-	1+
	24	0	9.2×10^2	0	2+	4+
	36	1+	4.8×10^3	16	2+	4+
	48	1+	2.8×10^4	32	2+	4+
	72	2+	1.2×10^4	64	3+	4+
	96	3+	1.0×10^4	32	3+	4+
	120	4+	7.5×10^3	32	2+	4+
	144	4+	2.5×10^3	16	1+	4+
C6/36						
	1	0	1.2×10^4	0	-	-
	12	0	6.0×10^3	2	-	1+
	24	0	6.4×10^5	16	2+	4+
	36	1+	3.5×10^6	96	2+	4+
	48	2+	2.3×10^7	256	3+	4+
	72	3+	3.8×10^7	256	3+	4+
	96	3+	6.0×10^7	256	3+	4+
	120	4+	5.6×10^7	512	3+	4+
	144	4+	3.2×10^7	1024	3+	4+

1 CPE: 0, no cells showing CPE; 1+, 25% of the cells; 2+, 50%; 3+, 75%; 4+, 100%
2 plaque-forming units per ml
3 Mean hemagglutination titer of clarified tissue culture supernatants.
4 Immunofluorescence: 0, no cells showing fluorescence; 1+, 25% of maximum fluorescence; 2+, 50%; 3+, 75%; 4+, maximum fluorescence.

Identification of dengue virus isolates

Cultered mosquito cells have been reported to be better hosts for the isolation of arboviruses (20,21,22). In order to compare viral isolations in LLC-MK2 and C6/36 cells in our laboratory, isolates from Jamaican patients were inoculated onto both cell lines and harvested when 50% of the cells displayed CPE. Monoclonal antibodies (15F3, 3H5, 5D4, and 1H10) were used in the indirect immunofluorescence assay to serotype these isolates. As can be seen in Table 3, generally greater yields of these low passage viruses (mean difference=2.9 $\log_{10}$) could be obtained using C6/36 cells. Moreover, in those cases where acute human sera were used as sources of virus inocula (Table 3), C6/36 cells were better able to allow replication of the viral isolates. Confirmation of each serotype identity was made using the plaque-reduction neutralization test. Representative results are presented in Table 4.

Dengue isolates from African patients (Senegal) or trapped mosquitoes (Ivory Coast and Upper Volta) were serotyped using AP-61 cells and type-specific monoclonal antibodies. Eighty-four viral isolates from trapped mosquitoes were identified as DEN-2, and three isolates from dengue fever patients (one DEN-2, two DEN-1) were also identified. Representative results are presented in Table 5. Complement fixation tests performed by the Institute Pasteur, Dakar, had previously suggested that these isolates were dengue viruses (Dr. Digoutte, personnal communication). Confirmation of the identity of these isolates was done by PRNT of representative isolates as shown in Table 4.

DISCUSSION

The monoclonal antibodies characterized in this study can be divided into 4 major groups based on their reactions in the immunofluorescence assay: (1) flavivirus group reactive, (2) dengue complex specific, (3) dengue subcomplex specific, and (4) dengue serotype specific. Categories (1), (2), and (4) are compatible with subdivisions of the flavivirus group as suggested by others (5,23,24). The possibility of a serological relationship between DEN-1 and DEN-3 has been reported previously using complement fixation and neutralization assays (25,26); however, this is the first definitive evidence of a subcomplex existing among the dengue viruses. Previously, Trent (27,28) suggested that the major envelope glycoprotein (V3) of a flavivirus contains at least three determinants: flavivirus group reactive, complex specific, and serotype specific. He proposed that an invariant segment exists in the primary sequence of the envelope glycoprotein V3 and is the crossreactive determinant shared by all flaviviruses. The varible portion of the glycoprotein, which is the major portion of the molecule, contains epitopes which determine type specificity and complex specificity (27,28). The monoclonal antibodies in this study were prepared using methods which favor the development of monoclonal antibodies directed against structural proteins (17). These

TABLE 3

Serotype Identification by Indirect Immunofluorescence and First Passage Yields of Dengue Fever Patients From Jamaica, 1980-1981

Patient #	Inoculum	First Passage Virus Yields (pfu/ml) LLC-MK2	C6/36	Serotype Identification Indirect Immnofluorescence
AP-61				
247/80	3.0×10^2	8.0×10^2	6.4×10^6	DEN-1
1229/80	$<5.0 \times 10^1$	2.0×10^2	3.0×10^7	DEN-1
1462/80	2.0×10^2	6.0×10^1	1.0×10^7	DEN-1
1296/80	4.0×10^2	4.3×10^2	1.2×10^7	DEN-1
1376/80	<5	1.9×10^4	5.0×10^3	DEN-1
1525/80	2.7×10^2	3.5×10^3	4.3×10^6	DEN-1
1532/80	<5	$3.5 \times 10_1$	3.5×10^4	DEN-1
1533/80	<5	3.5×10^1	1.0×10^6	DEN-1
58/81	1.5×10^1	1.2×10^4	9.8×10^6	DEN-1
Human Serum				
1229s/80	$<5.0 \times 10^1$	<5	6.6×10^6	DEN-1
1462s/80	$<5.0 \times 10^1$	<5	3.4×10^4	DEN-1
1296s/80	$<5.0 \times 10^1$	1.2×10^3	8.2×10^6	DEN-1
1661s/81	<5	7.5×10^5	1.6×10^5	DEN-2
1726s/81	3.0×10^1	1.4×10^4	8.7×10^5	DEN-4
1776s/81	3.3×10^2	7.0×10^4	1.5×10^6	DEN-4
1870s/81	3.4×10^2	8.1×10^4	1.3×10^6	DEN-4
1943s/81	6.0×10^1	1.8×10^4	6.4×10^5	DEN-4
1903s/81	4.0×10^1	1.3×10^3	6.4×10^5	DEN-2
1902s/81	<5	<5	2.2×10^5	DEN-4

TABLE 4

Serotype Identification of Dengue Virus Isolates by Neutralization.

Isolate	Origin	Plaque Reduction Neutralization Titer			
		DEN-1	DEN-2	DEN-3	DEN-4
247/80	Jamaica	320	<10	12	<10
1462/80	Jamaica	>320	<10	22	<10
1666s/81	Jamaica	12	>320	12	20
1903s/81	Jamaica	<10	>320	<10	<20
1776s/81	Jamaica	<10	<10	<10	19
1870s/81	Jamaica	<10	<10	<10	18
A510	Africa	32	>320	22	10
891	Africa	25	>320	24	10
20761	Africa	24	>320	22	12

TABLE 5

Serotype Identification of African Dengue Isolates by Immunofluorescence Using Monoclonal Antibodies

Isolate #	Origin *	Serotype Identification
A510	A. taylori	DEN-2
2022	A. africanus	DEN-2
2010	A. luteocephalus	DEN-2
1035	A. opok	DEN-2
891	A. opok	DEN-2
20761	A. luteocephalus	DEN-2
10674	DF patient	DEN-2
29177	DF patient	DEN-1
28790	DF patient	DEN-1

A. = Aedes
DF = Dengue Fever.
* Information provided by Dr. J.P. Digoutte, Institut Pasteur, Dakar, Senegal

antibodies also were reactive in serological tests which are based on the presence of virion antigens (hemagglutination-inhibition and neutralization tests). We propose that a fourth epitope contained within the varible region of V3 must also exist which is complex specific and common to DEN-1 and DEN-3. This would be the first definitive description of a subcomplex category among the dengue viruses. Moreover, monoclonal antibodies 9D12 and 2H2 provide serological confirmation of the dengue complex, because they were totally unreactive with the other flaviviruses which we examined.

This study demonstrated that serotype specific monoclonal antibodies prepared against high passage laboratory strains also can be used successfully to serotype low passage isolates from clinical or field environments. The ability of these unique antibodies to serotype virus strains with widely different passage histories (some separated by almost 25 years) is evidence of the immutability of the type specific determinant with which these antibodies react.

In order to determine the best host cell for demonstrating fluorescence with the dengue viruses, a comparison was made in this study between LLC-MK (monkey kidney) and C6/36 cells. Both cell lines proved suitable for these fluorescence studies using highly passaged DEN-2 (new Guinea C) virus at a multiplicity of infection equal to 1 plque forming unit per cell. Both had detectable dengue antigen (using monoclonal antibodies) within 24 hours and developed maximum fluorescence within 48 to 72 hours postinfection. However, the C6/36 cell, in agreement with reports by others (20,21,22) was a superior host for dengue viruses, yielding an average of about 3 $\log_{10}$ more infectious virus. Its superiority as a medium for dengue virus isolation was further substantiated by the significant amplification of low-titered virus inocula to easily detectable levels (Table 3).

For this study, infected cells were not harvested until 50% of the cells displayed CPE to insure a sufficient level of the single determinant against which these monoclonal antibodies were directed. This level of CPE often occurred 9 to 14 days p.i. or was difficult to detect in C6/36 cells in the absence of polykaryocyte formation. Even so, serotype identification using monoclonal antibodies often preceded PRNT identification by 14 to 21 days. Evidence obtained using DEN-2 (New Guinea C) virus suggests that type-specific monoclonal antibodies could be used to serotype isolates even in the absence of CPE or viral HA (Table 1). It is proposed that significant time and resources could be saved if type-specific monoclonal antibodies are used in conjunction with C6/36 cells. The use of monoclonal antibodies in the manner described in this report by field laboratories should make dengue virus isolation and identification a rapid and routine procedure.

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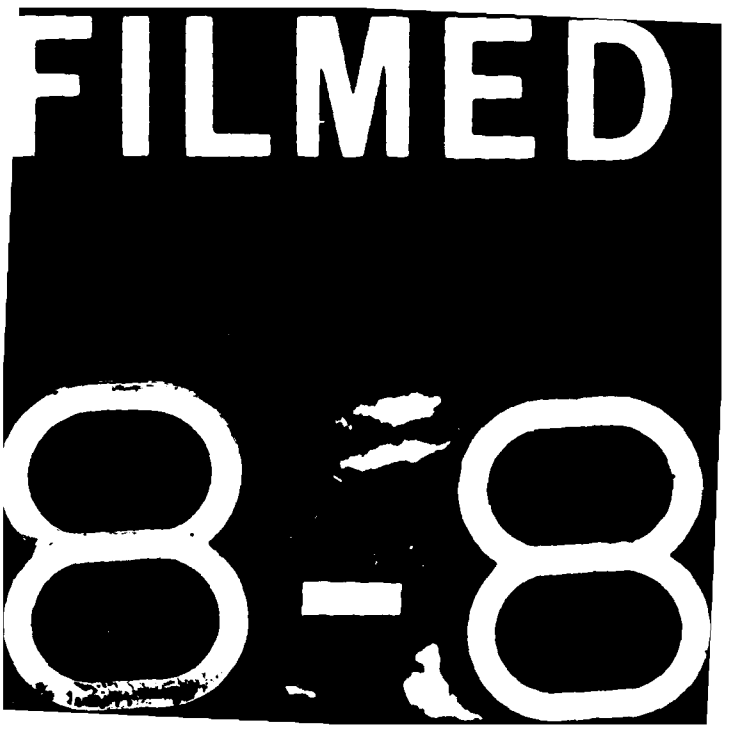
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